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REPORT DATE: June 20FF

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-06-2011		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 15 MAY 2010 - 14 MAY 2011	
4. TITLE AND SUBTITLE Participation of Bone Marrow-Derived Cells in the Formation of Tumor-Associated Stroma During Lung Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-0363	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Yongbaek Kim E-Mail: ykim3@ncsu.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) North Carolina State University Raleigh, NC 27695				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Tumor-associated stroma is known to play a critical role in the formation and progression of lung tumors. This study was performed to evaluate the fate of bone marrow derived cells (BMDCs) in the normal and neoplastic lung tissues. We have successfully established several experimental protocols which are critical to pursue the main objective. These include protocols for lung cancer development using adenovirus and conditional knockout mice, BMDCs isolation and culture from mice, transplantation of the BMDCs by irradiating mice. We confirmed the migration and distribution of BMDCs in the stroma of normal lung tissues. The fate of BMDCs in the lung tumor tissues are under investigation. Successful completion of the proposed study will provide a novel experimental system which can be used to study mechanisms that control the formation of the tumor-associated stroma, critical interactions between bone marrow-derived cells and tumor cells and/or resident stromal cells, and treatments that control stromal cells and, thus, tumor growth.					
15. SUBJECT TERMS Lung tumors, bone marrow stem cells, tumor stroma, conditional knockout mice					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 9	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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1. Introduction

Lung cancer accounts for 30% of annual cancer deaths (170,000 deaths in the United States), and is the second most frequently diagnosed cancer in men and women. Lung tumors typically arise from the epithelium of conducting airways or alveolar parenchyma. Tumor cell survival and growth is thought to be in part dependent on tissue microenvironments provided by stromal cells. In the last decade, several cell populations in the bone marrow were suggested to form TAS; two of these are mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs). The BMDCs can be mobilized into the bloodstream, and are then recruited to tumors, promoting tumor growth and progression. Yet, whether or not BMDCs are the source of TAS remains controversial. While several studies have shown significant contribution of BMDCs to tumor vascularization, others have reported that the presence of BMDCs in TAS is artifactual rather than physiological. Furthermore, it is not known which cell fractions in BMDCs are involved in the process. Morphologically, the amount of TAS is significantly increased in advanced adenocarcinoma compared to earlier stage of carcinogenesis, indicating that TAS formation is significantly enhanced at the progression stage.

The goal of this study was to understand the fate and function of bone marrow derived cells (BMDCs) in the formation of lung tumor-associated stroma (TAS). To test the hypothesis that **a fraction of mesenchymal stem cells migrate from the bone marrow and participate in the formation of tumor-associated stroma at the progression stage of lung carcinogenesis**, following experiments were performed according to the approved Statement of Work.

2. Body

Task 1. Production of a microchimera mouse lung cancer model harboring GFP-labeled BMDCs.

Breeding pairs of conditional and GFP-expressing transgenic mice were obtained from NCI mouse repository and Charles River, Inc., respectively. These mice were transferred to and kept in Laboratory Animal Resources at The College of Veterinary Medicine, NCSU. In order to obtain enough number of mice for the studies, we bred the mice and genotyped the progenies as below.

1.A. Animal breeding

1.A.1. Conditional knockout mice (K-ras^{G12d} /+ p53^{fl/fl})

Number of original breeder pairs: 3

Number of replacement breeder pairs: 5

Total Number of offspring: 98

K-ras^{G12d} /+ p53^{fl/fl}: 55% offspring

K-ras^{+/+} p53^{fl/fl}: 45% offspring

Genotyping

DNA was purified from Mouse Tail Tissue using the Qiagen Gentra Puregene Mouse Tail Kit. The genotype of the mice was determined by PCR reaction with Kras F1, Kras R1 and SD5' primers. Primers were ordered from IDT based on sequence information from Dr. Kirsch lab.

Wild Type: Kras F1 5'- GTC TTT CCC CAG CAC AGT GC -3'

Kras R1 5'- CTC TTG CCT ACG CCA CCA GCT C -3'

Mutant: SD5' 5'-AGCTAGCCACCATGGCTTGAGTAAGTCTGCA-3''

Kras R1 5'- CTC TTG CCT ACG CCA CCA GCT C -3'

Mutant Kras =~550bp

Wild type Kras=~ 650bp

PCR was performed using 1X PCR Buffer, dNTP (0.2mM), MgCl₂ (0.7 to 1.4 mM), Primers (0.4uM), Taq polymerase (1.9 units per reaction) and DNA (1ul per sample)

PCR conditions were an initial denaturation at 95 ° C for 2min followed by 34 cycles of denaturation at 94 ° C for 30sec, annealing at 60 ° C for 1min 30sec and extension at 72 ° C for 1min, followed by final extension at 72 ° C for 10 min The PCR was performed using the Applied Biosystems Gene Amp PCR system 2400 machine

1.A.2. GFP expressing transgenic mice

Number of breeder pairs: 2 original breeder pairs and 2 replacement breeder pairs

Breeding scheme: ++ female x hemizygote male

Litter size: 6-7 pups

The above mice were used for developing the bone marrow transfer protocol, as donors for tail vein injections, for the long term culture of bone marrow cells, developing the immunohistochemical procedure and as positive controls for analysis of chimeras.

1.B. Established protocols for isolation and culture of BMDCs.

Donor Mouse Strain: C57BL/6-Tg(CAG-EGFP)131Osb/LeySopJ Age: 6weeks

Euthanized the donor mouse and placed in 70% ethanol. The skin was removed to expose musculature and an incision was made beneath rib cage. Both femurs and tibia were collected from donor and as much hair, skin and muscles was removed as possible. The bones were placed in culture media {RPMI 1640 + 2% FBS + 10 units/ml heparin and antibiotics} in small culture dish. All attached connective tissue was removed and bones were stored on ice. The epiphysis and distal edges of each bone was clipped, the bone marrow was flushed out using the above media and a 27 gauge needle. The clumps were agitated by passing marrow through a 23 gauge needle to break up any clumps. The suspension was then strained through a 40um cell strainer. The cells were collected and centrifuged 2000rpm 8min at room temperature. The pellet was resuspended in RPMI 1640 + 2% FBS + 10 units/ml heparin and antibiotics and cells counted. Cells were resuspended in serum free RPMI.

1.C. Established protocols for lethal and sublethal irradiation and transplantation

A week prior to irradiation, mice were started on antibiotic water and Nutragel diet. Ten days following viral infection with AdCMVCre virus the mice were placed in autoclaved radiation disc (Braintree scientific) and transported for radiation. All eight mice were irradiated with dosage of 12 Gy (one time) using Varian Clinac 1800 with 6 MV photon energy. The mice were returned to their cages with Nutragel diet and antibiotic water.

Task 2. Analysis of lung tumor tissues for the distribution of BMDCs.

2A. Collected lung tumor samples representing different carcinogenic stages from the microchimera mouse lung cancer model.

2.A.1: Production of lung cancer model: Preparation and infection of Ad-Cre using the intranasal infection method

The Ad5CMVCre virus was ordered from Gene Transfer Vector Core, University of Iowa. Mice were infected between 7-12 weeks of age.

After the adenovirus was thawed on ice, 2.5ul of virus is pipetted directly into 125ul of MEM to obtain a final titer of approximately 5.5 to 7.5×10^6 PFU per mouse. The tube was mixed and 0.6ul of 2M CaCl_2 was added into the MEM-virus mix to obtain a final CaCl_2 concentration of 10 mM. The mix was incubated at room temperature for 20 min to allow for the formation of calcium phosphate precipitates.

Mice were anesthetized via intra-peritoneal injection of a mixture of 1 ml Ketamine (100 mg/ml), 1 ml Xylazine (20 mg/ml) and 5 ml sterile PBS at 3 ml/Kg body weight. In the biosafety hood, the mouse was placed in the hand, ventral side up. The mouse was tilted so that the head is positioned above its feet. Holding the end of a pipet tip over the opening of one nostril the virus was dispensed dropwise until the entire volume of virus had been inhaled. The mouse was placed on a recirculating warm water pad to recover. Mice were infected with a volume ranging from 50 - 75 μl per mouse.

2.A.2: Collection of lung tumor and tissue samples and *Histopathological evaluation of the lung tumors.*

Total number of mice infected: 17 mice
KP (K-ras^{G12d} /+ p53^{fl/fl}) genotype= 12 mice
P (K-ras^{+/+} p53^{fl/fl}) genotype= 5 mice

Liver, Lung, kidney spleen heart, femur tibia and ear clip were collected and fixed in 10% buffered formalin. Pieces of the lung and earclip are frozen at -80C.

ID	GENOTYPE	SEX	EUTHANIZE ON	WEEKS POSTINFECTION
3491	KP	M	April 25, 2011	12
3488	KP	F	April 25, 2011	12
3490	KP	F	April 25, 2011	12

3489	P	F	April 25, 2011	12
3496	P	F	April 25, 2011	12
3492	P	M	April 25, 2011	12
3495	P	M	April 25, 2011	12
3524	KP	M	July 28, 2011	16
3529	KP	M	July 28, 2011	16
3530	KP	F	July 28, 2011	16
3528	P	F	July 28, 2011	16
3559	KP	M	Sept 5, 2011	12
3566	KP	M	Sept 5, 2011	12
3568	KP	M	Sept 5, 2011	12
3593	KP	M	Oct 3, 2011	16
3562	KP	F	Oct 3, 2011	16
3582	KP	F	Oct 3, 2011	16

Samples from the April 25th batch and July 28, 2011 batch have been collected. The April 25th batch has been sectioned and analyzed. Based on H and E staining lung tumors were obtained in the KP genotype mice.

2.B: Evaluation of the fate of BMDCs in the mouse lung cancer model.

One day following irradiation of the viral infected mice, seven were injected with 7.5×10^6 donor GFP bone marrow cells via the lateral tail vein. However, because the mice lost weight less than 15% of original body weight, all the mice were euthanized within 14 days post-innoculation of BMDCs via tail veins.

SNO	ID	Sex	Age at infection (weeks)	Age at tail vein injection (weeks)	Tail vein injected	Euthanized (days after tail vein injection)
1	3541	F	10	12	yes	Day 9
2	3503	F	10	12	yes	Day 9
3	3507	F	8	10	yes	Day 13
4	3515	F	7	9	yes	Day 14
5	3538	F	10	12	yes	Day 7
6	3539	F	8	10	Could not get cells in when injected	Day 3
7	3540	F	8	10	Died at injection	Day 0

2.B.1: Histopathological evaluation of the tissue samples.

The recipient mice were euthanized at different time points within 14 days of injection. The lung, liver spleen, kidney and heart along with femur and tibia were collected and placed in formalin. Tissues were embedded in paraffin and sections were evaluated with the GFP antibody and H and E staining.

Findings:

- Bone marrow samples of the tibia were significantly hypocellular, indicating the effective bone marrow cell depletion in control mice with no injection of BMDCs.

- Bone marrow samples of the tibia were normocellular, indicating the effective replacement of bone marrow cells with transplanted BMDCs.

2.B.2: Immunohistochemistry with the GFP antibody

Formalin-fixed, paraffin-embedded tissues were sectioned at 3-5 um thickness, mounted on glass slides, baked in an oven at 60°C for one hour, deparaffinized in xylene, and rehydrated through a graded series of ethanol concentrations. Antigen retrieval was performed with the target retrieval buffer in the DakoCytomation Pascal pressure chamber according to manufacturer's recommendations. Following antigen retrieval, slides were placed in running tap water and used for immunohistochemistry.

Immunohistochemical staining was performed using the Vectastain ABC Kit (Vector Laboratories) based on manufacturer's recommendations. Endogenous peroxidase activity was quenched by incubation in Hydrogen peroxide (3%) solution in water for 10 or 30 minutes. Primary antibody used in this study is a rabbit polyclonal to GFP (abcam ab6556 at 1:1000 dilution). As a negative control, the primary antibody was replaced with normal serum. Tissues obtained from mice that were not injected with GFP cells and incubated with the primary antibody were also used as a control. As a positive control, we used tissue from C57BL/6-Tg(CAG-EGFP)131Osb/LeySopJ mice. Staining was visualized by adding DAB Substrate (Vector Labs) to the sections for 1 min. Sections were rinsed and counterstained with Mayer's Hematoxylin and coverslipped with Permount.

Findings:

By immunohistochemical staining, the positive staining for GFP was detected predominantly in the connective tissue around the smaller bronchioles.

2.C. On-Going experiments (Approved No Cost Extension)

2.C. 1. Adenoviral infection, irradiation and transfer of BMDCs 8 weeks postinfection with Ad5CMVCre virus

The following seven mice were infected with Ad5CMVCre virus according to above protocol. At 8-10 weeks post infection these mice will be irradiated with dosage of 10 Gy (one time) using Varian Clinac 1800 with 6 MV photon energy. One day following irradiation of the viral infected mice, they will be injected with 7.5×10^6 donor GFP bone marrow cells from C57BL/6-Tg(CAG-EGFP)131Osb/LeySopJ mice via the lateral tail vein.

ID	GENOTYPE	SEX
3606	KP	M
3614	KP	M
3615	KP	M
3616	KP	M
3609	KP	M
3618	KP	F
3619P	P	F

2.C.2. Microscopic Evaluation:

The recipient mice will be euthanized at different time points. The lung, liver, spleen, kidney and heart samples along with femur and tibia will be collected and placed in formalin. Tissues will be embedded in paraffin and sections will be used for immunohistochemical analysis with the GFP antibody.

3. Key Research Accomplishments

- a. Established lung cancer model using adenovirus and conditional knockout mice.
 - Obtained lung cancer tissues
- b. Established protocols:
 - Culture of mouse bone marrow stem cells
 - Transplantation of bone marrow cells by irradiating mice
- c. Determined the fate of bone marrow cells in the lung tissues and tumors: Under investigation.

4. Reportable Outcomes

- Plan to present the results in a scientific meeting.

5. Conclusion

This award was used to evaluate the fate of BMDCs in the normal and neoplastic lung tissues. The results illustrated that the BMDCs migrate to the lung tissues, indicating their participation in the formation of lung TAS. Currently, migration and distribution of the BMDCs in the lung tumor tissue of microchimeric mouse model is under investigation, with the approved No Cost Extension (effective until July 14, 2012).

Proposed experiments to reveal which of the BMDC fraction is the source of stromal cell components in lung tumors cannot be preformed due to the severity of irradiation toxicity, small litter size than expected and high mortality rate of progenies from conditional knockout mice, and resultant shortage of the funding.

Although it can be partially completed, this study provides a novel experimental system which can be used to study mechanisms that control BMDC migration, interactions between BMDCs and tumor cells and/or resident stromal cells, and treatments that control stromal cells and tumor growth.

6. References

7. Appendices